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The optimization of the Ambonese arrack fermentation using co-culture *Pichia polymorpha* and *Kloeckera javanica*

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Abstract. *Mahulette F, Astuti DI. 2020. The optimization of the Ambonese arrack fermentation using co-culture* Pichia polymorpha *and* Kloeckera javanica. *Biodiversitas 21: 2900-2906.* The fermentation process of the Ambonese arrack is still uncontrollable. As various microbes are involved in the fermentation, which causes inconsistent production of ethanol content. This research aimed to optimize the production of the Ambonese arrack using *Pichia polymorpha* and *Kloeckera javanica* in inoculum ratios of 1:1, 1:2, and 1:3. The number of yeast cells and chemical characteristics were observed every three hrs up to 24 hrs. The ethanol content of the fermented coconut sap and the arrack was measured using the titration and Gas Chromatography-Mass Spectrophotometry (GC-MS) methods, respectively. The numbers of *P. polymorpha* and *K. javanica* cells at 15 hrs of fermentation were 9.9 log CFU/mL and 10.7 log CFU/mL, respectively. The reducing sugar content decreased from 525 mg/L to 296 mg/L, while the pH from 6.46 to 4.82. The highest ethanol production rate was 1.4 mg/L.3hrs. (ratio 1:1), observed at 12 hrs of the fermentation, while the highest ethanol contents in the fermented coconut sap and the arracks were 11.3 mg/L and 300.400 mg/L, respectively. Different inoculum ratios affect the sensory characteristics of Ambonese arrack. The highest average value of sensory testing was 4.6 (ratio 1:2). Thus, the inoculum ratios of *P. polymorpha* and *K. javanica* 1:2 and 1:3 has the best quality in controlled Ambonese arrack processing.

Keywords: Ambonese arrack, coconut sap, ethanol content, reducing sugar content, sensory characteristics

INTRODUCTION

Ambonese arrack (locally known as sopi) is an alcoholic beverage produced from distilled fermented saps (locally known as sageru). The beverage is consumed by Moluccans in eastern Indonesia. The saps are from various palm plants, such as coconut (*Cocos nucifera* L), sugar palm (*Arenga pinnata* Merr), and koli palm (*Borassus sundaicus* Becc). The processing of arrack using the sap of koli palm is only done by the Kisar Community in Southwest Moluccas (Bartels 2017). Ambonese people use coconut sap more to process arrack than other plants. The arrack is available in sufficient quantities in the local market and the consumption of which is not limited by social status (Ririhena et al. 2015). It also serves as a symbol of *pela-gandong*, one of the social relationships among villages in Moluccas (Titaley et al. 2018).

The Ambonese arrack is still produced traditionally, and the fermentation takes place spontaneously. In the process, because various indigenous microbes, especially yeasts are involved, the quality of the beverage becomes inconsistent. The fermentation of Ambonese arrack also involves many bacterial contaminants that are dominated by *Bacillus* sp. (Mahulette and Astuti 2020). The indigenous yeasts are presumably more competitive than the commercial ones as they adapt better against the local ecological conditions and may potentially dominate the fermentation process (Udomsaksakul et al. 2018). The fermentation of the Ambonese arrack is dominated by *Pichia polymorpha* and *Kloeckera javanica* (Mahulette and Astuti 2020). Both are of indigenous yeast genera (wild types) found in the production of alcoholic beverages (Buzzini et al. 2017). *Pichia* generally uses more sucrose, while *Kloeckera* uses fructose to grow (Kalaiyarasi et al. 2013). Therefore, it is expected that the combination of these two yeasts is capable of increasing ethanol production in the arrack fermentation.

The fermentation process of alcoholic beverages using non-*saccharomyces* starters has been explored to obtain potential yeasts, one of which is *Pichia* (Kaur et al. 2019). *P. polymorpha* and *K. javanica* are also found in the tavern (Cautino et al. 2020) and toddy (Santiago-Urbina et al. 2014), fermented beverages of Sri Lanka. The use of co-culture starters in fermentation is capable of producing higher final ethanol content than one starter only (Halim and Faizal 2014). Selection of microbial starter in processing of alcoholic beverages is not only to maximize the alcohol yield, but also to maintain beverage sensory quality (Walker and Stewart 2016). This research aimed to optimize the controlled fermentation of the arrack using *P. polymorpha* and *K. javanica* as starters. It is expected that the results of this study are capable of improving the quality of the Ambonese arrack as the traditional beverage of the Moluccans.

MATERIALS AND METHODS

Preparation of coconut sap and inoculum

A total of 150 mL of coconut sap was obtained from a coconut sugar producer in Cipatujah, West Java. After pasteurization, the coconut sap was put into three 500 mL Erlenmeyer flasks and inoculated with *P. polymorpha* and *K. javanica* as starters in ratios of 1:1, 1:2, and 1:3. Both yeasts were previously isolated from the traditional fermentation of the arrack (Figure 1). Compared to *P. polymorpha*, *K. javanica* inoculum was observed more abundant as the latter is the dominant microbe in the traditional fermentation of the Ambonese arrack (Mahulette and Astuti 2020), so that the ratio of this yeast inoculum was higher than *P. polymorpha*. The number of yeast cells and chemical characteristics were observed every three hours for 24 hours.

The enumeration of the yeast cells

The number of yeast cells was calculated using a hemocytometer following the improved Neubauer method. A total of 1 mL of sample was put into a test tube prior to the addition of two drops of methylene blue. After the color dissolved, two drops of the sample were added onto the counting chamber. The total yeast population was calculated under a microscope with 400x magnification. Blue-colored cells were not counted. Only five out of the 25 squares in the middle quadrant were used for the counting. The total number of yeast was calculated following the formula: cells/mL = total cells in 5 squares x 50,000/dilution factor (Salares and Balala 2018).

Analysis of chemical characteristics

To measure the reducing sugar content, a total 2 mL sample was mixed with 1.6 mL Somogyi I and 0.4 mL Somogyi II solutions and allowed in a water bath for 10 minutes. The mix was then cooled in the ice for 5 minutes prior to the addition of 2 mL Nelson solution and 4 mL distilled water. The absorbance was measured at 520 nm wavelength (Barlianti et al. 2015) and the results were expressed into mg/L following a standard glucose curve.

In addition, the ethanol content was measured following the redox titration method (Asakai and Hioki 2011). A total of 10 mL dichromic acid solution (0.01 M $K_2Cr_2O_3$ in 5M H_2SO_4) was taken into a 250 mL Erlenmeyer flask. One milliliter of sample diluted 10 times was pipetted into a small vial tube and placed above the Erlenmeyer flask. The flask mouth was covered prior to incubation for 18 hours at room temperature. After the incubation, the liquid sample in the small vial tube was removed. A total of 100 mL distilled water and 1 mL of 1.2M potassium iodide (KI) was added into the flask and shaken until the color turned brown. The solution was titrated with 0.03M sodium thiosulfate ($Na_2S_2O_3$) until the color turned yellow. 1 mL of 1% starch solution was added until the color turned dark blue. Finally, the solution was titrated again with sodium thiosulfate until the color turned clear light blue. The ethanol content (g/L) was calculated by following formula:

Ethanol (g/L) = [Vol (B-S)/1000] x [(mol thiosulfate x M_r.ethanol x dilution)/4

Where; Vol. B was the volume of $Na_2S_2O_4$ used in the blank titration and Vol. S was the volume of $Na_2S_2O_4$ used in the sample titration. At the end of fermentation, the fermented sap was distilled for 1 hour to obtain Ambonese arrack and the ethanol content was measured using a Gas Chromatography-Mass Spectrophotometer (GC-MS). The pH was measured using a pH meter (CT-6022, Shenzhen Kedida Electronic, China).

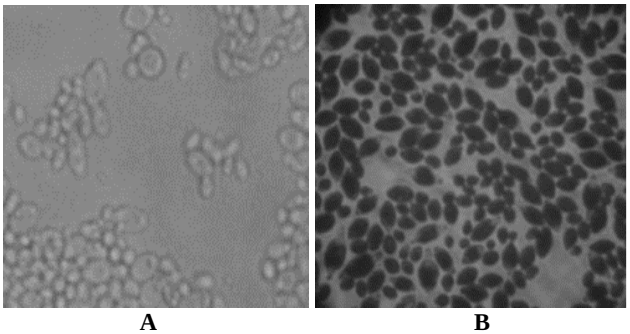
Analysis of sensory characteristics

Sensory analysis was carried out after the arrack was bottled using a multiple comparison test. Three types of Ambonese arrack produced with different inoculum ratios compared to traditional Ambonese arrack as control. The arracks were evaluated by 15 panelists. They were selected based on interest and availability as well as their experience in arrack sensory analysis. A total of 5 mL samples were presented in arrack glasses labeled with random codes. After smelling and testing the arrack, panelists marked the intensity of each chosen attribute on a 1 to 5 scale. Sensory description of arrack includes fruitiness, acidity, and bitterness. The panelists rinsed their mouth with water before being allowed to assess the next sample. Data were then analyzed using factorial analysis of variance (ANOVA). If the significant effect was found at a 95% confidence interval, ANOVA was followed by Duncan's multiple range test to identify differences among groups (Lorrain et al. 2012).

RESULTS AND DISCUSSION

The growth of the yeasts during fermentation

The growth of *P. polymorpha* and *K. javanica* was initially uniform, except for the ratio 1: 3. Both yeasts grew very slow until 9 hrs of the fermentation. Yeasts grow slowly at the beginning of the fermentation due to plasmolysis as the reducing sugar content is still high (Hashem et al. 2014). The growth increased at 9 to 15 hrs of the fermentation as the coconut sap contained a lot of nutrients (Asghar et al. 2019) used to increase the number of cells. The highest *P. polymorpha* and *K. javanica* growth were 9.9 log CFU/mL (ratio 1:2) and 10.7 log CFU/mL (ratio 1:3) respectively, at 15 hrs of the fermentation (Figure 2). Such numbers are sufficient to produce alcohol in the fermentation process (Kismurtono 2012).



A **B**

Figure 1. The dominant microbes in the traditional fermentation of the Ambonese arrack. A. *P. polymorpha*, B. *K. javanica* (400x magnification)

After 18 hours of fermentation, the growth decreased due to the declining nutrient supply and the accumulation of alcohol and other toxic compounds (Teixeira et al. 2012). At the end of the fermentation, the number of *K. javanica* cells was generally lower than *P. polymorpha* except for ratio of 1:3. *Kloeckera* is less tolerant against high alcohol content compared to *Pichia* (Waites 2005) whose enzymes are tolerant against high sugar and ethanol contents (Madrigal et al. 2012).

Chemical characteristics Ambonese arrack during fermentation

In general, the reducing sugar contents in all ratios decreased uniformly because both *P. polymorpha* and K. *javanica* assimilated the sugar in the coconut sap to produce energy. In the beginning, the reducing sugar contents of the ratio 1:1, 1:2, and 1:3 were 525 mg/L, 521 mg/L, and 517 mg/L, respectively, before they decreased to 311 mg/L, 309 mg/L, and 296 mg/L, respectively (Figure 3.A). The decrease was not significant at the beginning as the yeast required time to secrete invertase enzyme in cells. Yeast generally has invertase enzyme, including *Pichia* (Kadowaki et al. 2013). Invertase is an enzyme that breaks down sucrose in the coconut sap into glucose and fructose. The simple sugars are reacted with another enzyme, zymase, in the yeast to produce ethanol (Offiong and Akpan 2017). At this stage, the yeasts used simple sugars in the coconut sap, and therefore, the reducing sugar content decreased. Generally, *Pichia* uses more sucrose, while *Kloeckera* uses more fructose, to grow (Kalaiyarasi et al. 2013).

After hour 6 of the fermentation, the invertase enzyme actively broke down sucrose into simple sugars, increasing the reducing sugars at 9 hrs of the fermentation. After which, as the growth of yeast accelerated, the reducing sugar decreased again until the end of fermentation. The increasing number of yeast cells improves its ability to reduce sugar (Kismurtono 2012). *K. javanica* preference to use fructose caused the reducing sugar content of ratio 1:3 decreased more than the other ratios.

The ethanol contents of the three ratios were found increasing uniformly. At the end of fermentation, the ethanol content of the ratio of 1:1, 1:2, and 1:3 was 9.1 mg/L, 11.0 mg/L, and 11.3 mg/L, respectively, (Figure 3.B). The highest ethanol production rate was 1.4 mg/L (ratio 1:1), observed at the hour 12 of the fermentation. The highest ethanol production in sap fermentation usually takes place 11 hours after fermentation (Shetty et al. 2017). In addition, glucose and sucrose are simple sugars quickly converted into ethanol (Offiong and Akpan 2017).

Generally, the ethanol production was very slow mid-fermentation because *K. javanica* had already reached its stationary phase. Although the growth is inhibited by high ethanol contents, the yeast is capable of producing higher ethanol content than *P. polymorpha* (Plata et al. 2003). In the ratio 1:3, the number of *K. javanica* cells was higher than *P. polymorpha,* causing the ratio produced more ethanol content than other ratios. The ethanol content continued to increase until the end of fermentation. This suggests that the ethanol could not be utilized as a carbon source under anaerobic conditions (Lin et al. 2012) and the fermentation process can still continue for more than a day.

The pH of the fermented coconut sap continued to decrease during the fermentation. At the end of the fermentation, the pH of the ratio 1:1, 1:2, and 1:3 dropped from 6.46 to 4.94, 4.82, and 4.88, respectively (Figure 3.C). pH above 4.0 can be considered an operational limit for an ethanol production process (Lin et al. 2012). The pH of the ratio 1:2 and 1:3 was lower than the ratio 1:1 because K. javanica is the best producer of acid compounds, including acetic acids, among yeasts (Plata et al. 2003). The pH was slightly increased at the end of fermentation because the yeast had reached a stationary phase and the ethanol production was already high.

The characteristics of Ambonese arrack

After 24 hours, the fermented coconut sap was distilled into Ambonese arrack. Its highest ethanol content (distillate) was 300.400 mg/L (ratio 1:3), followed by 289.600 mg/L and 279.000 mg/L for ratio 1:1 and 1:2, respectively (Figure 4.A). The lowest pH of the arrack was 3.44 (ratio 1:3) (Figure 4.B). The ethanol content of the arrack was higher than the fermented sap because the water content in the fermented sap was still high. The ethanol from the fermentation process contains some water to be removed through the fractional distillation process (Offiong and Akpan 2017). The ethanol content of the Ambonese arrack from the distillation of the fermented coconut sap in ratio 1:3 was found the highest because the ratio comprised way more *K. javanica* than *P. polymorpha.*

Sensory characteristics of Ambonese arrack

Sensory analysis of three types of Ambonese arrack from controlled fermentation with traditional Ambonese arrack (Figure 5.A) showed that highest value of Ambonese arrack was produced using inoculum ratio of *P. polymorpha* and *K. javanica* 1:2, while lowest value was recorded in 1:1 ratio (Figure 5.B). Non-*saccharomyces* yeast strain can improve product quality of arrack. During the fermentation, these yeasts release secondary products such as higher alcohols, esters, acids, carbonyl compounds that important to the sensory characteristics of arrack. Some of these strains such as *Pichia* and *Kloeckera* have been studied for their organoleptic contributor (Mendoza and Farias 2010).

Kloeckera can produce a large high amount of acetate esters, such as phenylacetate and ethyl acetate. These compounds are condensation of alcohol with acetate. *Kloeckera* is the highest producer of acetate (Plata et al. 2003). These ester compounds contributed to the fruitiness aroma of alcoholic beverages (Martin et al. 2018). Pichia also produces volatile compounds that contribute to the aroma of Ambonese arrack, but the amount of compounds produced by this yeast is lower than *Kloeckera*. One of the volatile compounds produced by this yeast is ethyl lactate (Amaya-Delgado et al. 2013). Pichia also plays a role in the fermentation of cabernet sauvignon, a beverage from China (Li et al. 2010).

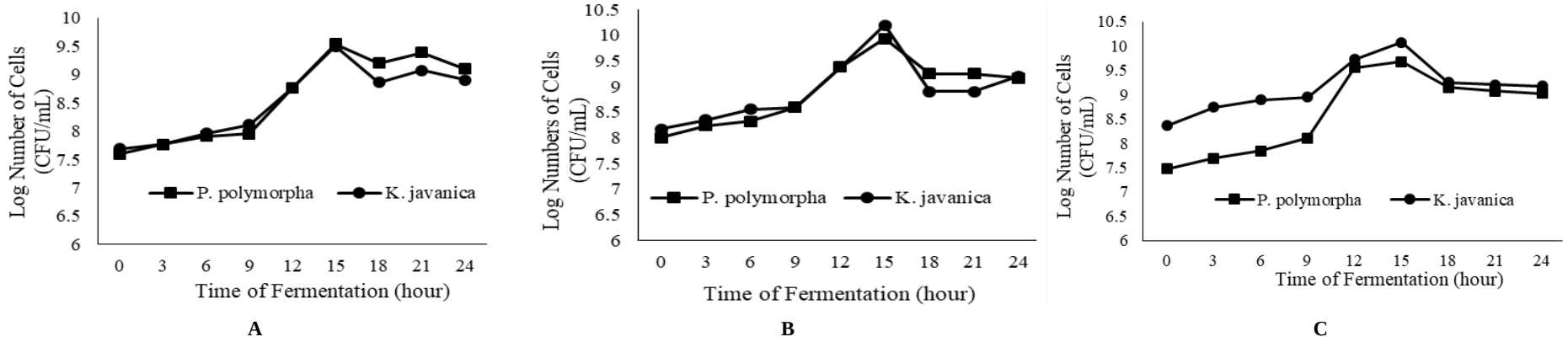


Figure 2. The growth of *P. polymorpha* and *K. javanica* in different inoculum ratios 1:1 (A), 1:2 (B), and 1:3 (C)

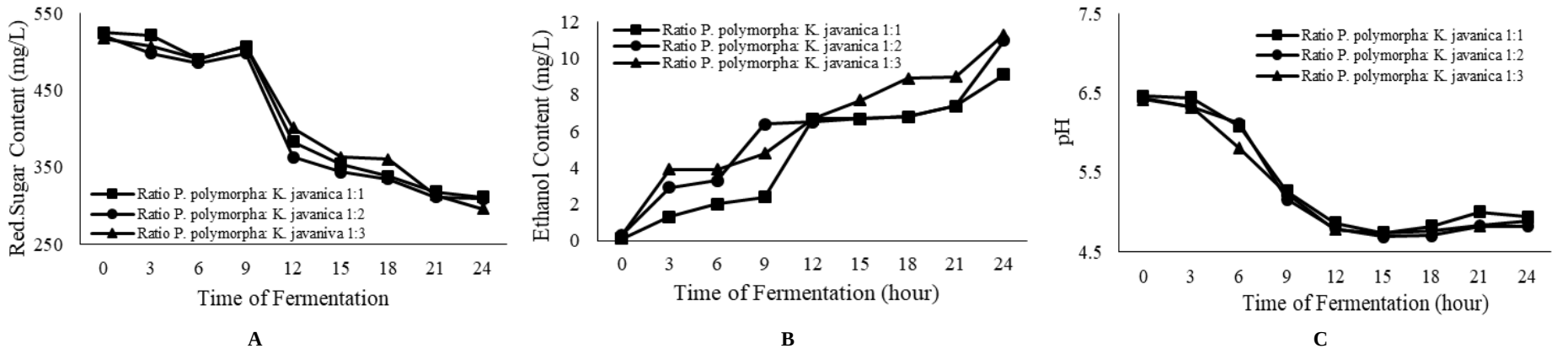
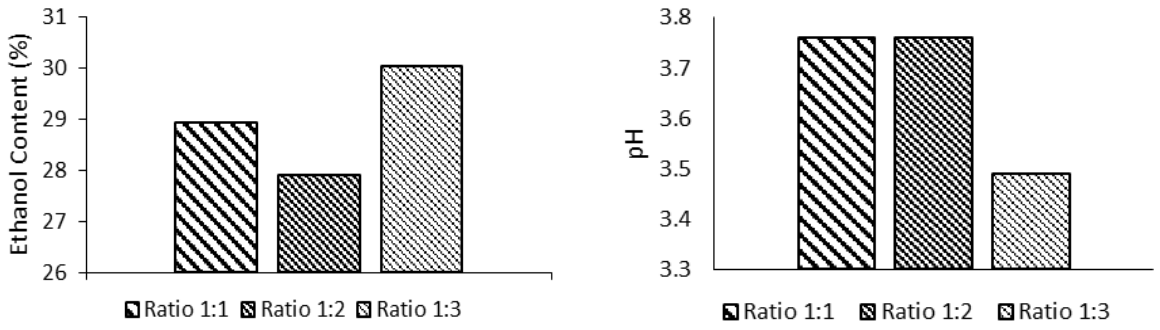
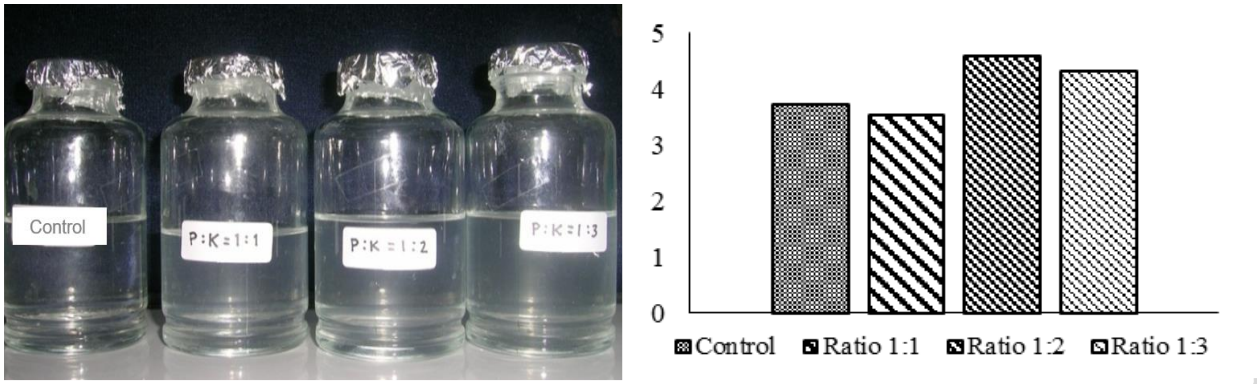


Figure 3. The change of chemical characteristics during the controlled fermentation of Ambonese arrack. Reducing sugar content (A), Ethanol content (B), pH value (C)



A **B**

Figure 4. The characteristics of the Ambonese arrack from the controlled fermentation (distillate). Ethanol content (A), pH value (B)



A **B**

Figure 5. Traditional and controlled fermentation of Ambonese arracks (A), Average of sensory test with 15 panelists (B)

The results of ANOVA with different inoculum ratio treatments were great effect on sensory characteristics of Ambonese arrack (Table 1). The result of Duncan's multiple range test at level of 5% showed that the Ambonese arracks produced with inoculum ratios of 1:2 and 1:3 found different from traditional Ambonese arrack and inoculum ratio 1:1 (Table 2). Traditional Ambonese arrack has an unpleasant aroma, so it is less preferred by panelists. The contaminant microbes in traditional fermentation of Ambonese arrack have the enzyme cysteine desulfurase, which can break down the cysteine into hydrogen sulfide (Albrecht et al. 2011). Hydrogen sulfide (H_2S) is a compound that reduces the sensory quality of traditionally fermented beverages (Bekker et al. 2016).

Table 1. One way ANOVA test result in sensory characteristics of Ambonese arrack

Source of variability	Degree of freedom	Sum of square	Mean square	F value	F Table	
					0.05%	0.01%
Samples	3	11.25	3.75	8.52**	2.34	4.31
Panelist	14	6.85	0.48	1.09	1.92	2.52
Error	42	18.75	0.44			
Total	59	36.85				

Note: ** Significance level 1%

Table 2. Duncan's test result in sensory characteristics of Ambonese arrack

Samples	Difference between treatments	Significance level
Control-ratio 1:1	3.73-3.53	0.20<0.48
Ratio 1:3-ratio 1:1	4.33-3.53	0.80>0.50*
Ratio 1:3-control	4.33-3.73	0.60>0.48*
Ratio 1:2-ratio 1:3	4.60-4.33	0.27<0.48
Ratio 1:2-control	4.60-3.73	0.87>0.50*
Ratio 1:2-ratio 1:1	4.60-3.53	1.07>0.52*

Note: * Significance level 5%

In conclusion, the fermentation process of the Ambonese arrack is still uncontrollable. The results of the optimization of the arrack production using *P. polymorpha* and *K. javanica* as starters in ratios 1:1, 1:2, and 1:3 revealed that the highest ethanol content was found in ratio 1:3 (11.3 mg/L for the fermented coconut sap and 300.400 mg/L for the arrack). During the fermentation, the reducing sugar content decreased from 525 mg/L to 296 mg/L, and the pH decreased from 6.46 to 4.82. Different inoculum ratios affect the sensory characteristics of Ambonese arrack. The highest average value of sensory testing was 4.6 (ratio 1:2). Thus, the ratio 1:2 and 1:3 for *P. polymorpha* and *K. javanica* were the best to improve the quality of the Ambonese arrack. These findings can be used by Ambonese arrack producers to improve the quality of the traditional beverage of the Moluccans.

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